



RESEARCH ARTICLE

High incidence of *Plasmodium knowlesi* malaria compared to other human malaria species in several hospitals in Malaysia

Lai, M.Y.¹, Rafieqin, N.¹, Lee, P.Y.@Lee, Z.¹, Amir Rawa, M.S.¹, Dzul, S.¹, Yahaya, N.², Abdullah, F.H.³, Othman, N.⁴, Jelip, J.⁵, Ooi, C.H.⁶, Ibrahim, J.⁶, Aung, M.⁷, Abdullah, A.H.⁷, Laili, Z.⁸, Lau, Y.L.^{1*}

¹Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

²Jabatan Kesihatan Negeri Pulau Pinang, 10590 Pulau Pinang, Malaysia

³Pusat Penyelidikan Klinikal, Hospital Sultanah Nur Zahirah, 20400 Kuala Terengganu, Malaysia

⁴Microbiology Unit, Department of Pathology, Hospital Sultanah Nur Zahirah, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Malaysia

⁵Ministry of Health Malaysia, 62590 Putrajaya, Malaysia

⁶Sarawak State Health Department, 03050 Kuching, Sarawak

⁷Hospital Kuala Lipis, 27200 Kuala Lipis, Pahang

⁸Hospital Raja Permaisuri Bainun, 30450 Ipoh, Perak

*Corresponding author: lauyeeling@um.edu.my

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ABSTRACT

Through the regional control programme, Malaysia has been successfully reducing the incidence of *Plasmodium falciparum* and *Plasmodium vivax* infections. However, the incidence of zoonotic malaria *Plasmodium knowlesi* infection is increasing and now has been the major cause of malaria in Malaysia especially Malaysian Borneo. The emergence of *knowlesi* infection has threatened the malaria elimination programme which the government aims to reduce the overall malaria infections by 2020. Unlike other benign human *Plasmodium* spp., *P. knowlesi* can cause fatal infections. The aim of this study was to determine the incidence and distribution of five human malaria parasites including *P. knowlesi* in Peninsular Malaysia and Malaysian Borneo. A total of 112 blood samples were collected from seven states and district hospitals in Peninsular Malaysia and Malaysian Borneo from year 2015 to 2016. The samples were examined by microscopy and further confirmed by nested PCR assay targeting 18S rRNA gene of *Plasmodium* spp. Following the nested PCR assays, a total of 54 (48.2%) samples were positive for *P. knowlesi* infections, 12 (10.7%) cases were positive for *P. vivax* infections, followed by 7 (6.3%) cases of *P. falciparum* and 4 (3.5%) cases of *P. malariae*. There were 3 cases (2.7%) of mixed infections (*P. knowlesi*/*P. vivax*). However, no cases were identified as *P. ovale*. A total of 32 (28.6%) cases were found as negative infections. Loop-Mediated Isothermal Amplification Assay (LAMP) was performed to confirm inconclusive results produced by microscopy and nested PCR. *P. knowlesi* showed the highest prevalence in Sarawak (n= 30), Sabah (n=13), Pulau Pinang (n=5) and Pahang (n=6). PCR and LAMP was not able to detect a large number of microscopy positive samples due to DNA degradation during storage and shipping. Among all the states involved in this study, the highest prevalence of *P. knowlesi* infection was found in Sabah and Sarawak.

Keywords: Malaria; *P. knowlesi*; PCR; molecular diagnostic; LAMP.

INTRODUCTION

Malaria is one of the alarming vector-borne diseases, transmitted via the bite of female mosquitoes belonging to the genus of *Anopheles* and currently affecting 219 million people worldwide with 435000 death cases as of 2017 (Pimenta *et al.*, 2015; World Health Organization, 2018). Conventionally, only four species of the causative agent for malaria are medically important: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, and *Plasmodium ovale*. Nevertheless, there is a rise of malaria cases due to fifth

species that is pathogenic to human, *Plasmodium knowlesi*, among Southeast Asia's countries (Alemu *et al.*, 2013; Singh *et al.*, 2004). *Plasmodium knowlesi* is initially a simian malarial parasite which later on was discovered to be a potent contributor to malaria cases in Malaysia, accounting for 50% of malaria cases in Sarawak (Foster *et al.*, 2014). The occurrence of malaria infections is associated with farming and deforestation activities. Malaysia harbours abundant of rainforests which are the possible conduit to the highest rate of malaria cases among aboriginal (Orang asli) minority group and soldiers (Koh *et al.*, 2004; Jamaiah *et al.*, 2005).

Immigration of undocumented migrant workers from countries that are endemic for malaria infection is one of the conduits for malaria transmission and re-introduction of malaria in Malaysia (Jeffree *et al.*, 2018). Besides, hot climate and humidity further facilitate the transmission of malaria (Rahman *et al.*, 1997). Malaria infections is primarily impacting poor and rural populations. Various interventional malaria control programs and activities have been conducted and the reduction in the cases of malaria was notifiable until the year of 2004 as the cases of *P. knowlesi* infections were reported. Despite of the first malaria case by *P. knowlesi* was reported from Peninsular Malaysia dated back in 1965, it is no later than the early 2000s that a large focus of human knowlesi infection occurred in Kapit, Sarawak (Chin *et al.*, 1965; Singh & Daneshvar, 2010). Since then, a greater number of malaria cases has been reported from Malaysia and *P. knowlesi* is the main cause of malaria. The incidence of *P. malariae/P. knowlesi* infections in Sabah was increased by more than 10-fold between 2004 and 2011 rendering significant increment of 59 cases to 703 cases (William *et al.*, 2013). *P. knowlesi* appears to be the most common *Plasmodium* spp. in Malaysian Borneo (Sabah and Sarawak) while Pahang has been recognised as the highest contributor of human knowlesi malaria cases in Peninsular Malaysia between September 2012 and December 2013 (Yusof *et al.*, 2014). *P. vivax* was observed to be the predominant causative agent of malaria in Selangor (Vythilingam *et al.*, 2008).

To track the number of malaria cases in Malaysia, this study aims to elucidate the geographical distribution of malaria cases across several hospitals in Malaysia from August 2015 until June 2016 and a prospective malaria surveillance study.

MATERIALS AND METHODS

Sample collections

A total of 112 samples were collected from year 2015 to 2016. The samples were collected from states and district hospitals in Peninsular Malaysia (Kuala Lumpur, Perak, Pahang, Pulau Pinang and Terengganu) and Malaysia Borneo (Sabah and Sarawak). These samples were collected from malaria infected patients. This ethical approval for this study was obtained from the Medical Ethics Committee of UMMC (reference number: 817.18) and Medical Research Ethics Committee (MREC) (reference number NMRR-12-949-11328).

Informed verbal consents were obtained during the blood collections.

Microscopy

Thin films were prepared according to Giemsa's staining and thick films were prepared by Field's staining (Warhurst & Williams, 1996). Slides were air dried and examined by experienced microscopists to identify the malaria parasites. Parasitemia was calculated either per 1,000 erythrocytes in the thin film at low parasitemia or per 200 white blood cells in the thick film. The slide was considered as negative if no parasites were seen in at least 100 high-power fields. DNA templates were extracted from the collected blood samples by using DNeasy Blood and Tissue Kit following the manufacturer's protocol (Qiagen, Hilden, Germany). The extracted DNA was served as the template for nested PCR.

PCR amplification

A nested PCR was performed for the malaria infected samples, which targets on *Plasmodium* small subunit ribosomal RNA (18S rRNA) gene. The primers used for the assay have been previously described by Snounou *et al.* (1993) and Imwong *et al.* (2009) (Table 1). Four µL of the extracted DNA was used as template. Nested 1 PCR was performed in a final volume of 25 µL containing 1X of PCR buffer, 0.4 mM of each primer (rPLU1 and rPLU5) (MyTACG Bioscience Enterprise, Malaysia), dNTPs mix (200 µM), MgCl₂ (4 mM) and *Taq* DNA polymerase (1U) (Promega Corporation, Madison, United States). Cycling parameter was consisted of denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min.

Nested 2 PCR amplification is a species identification PCR assay, which consisted of 5 PCR assays, targeted on *P. knowlesi*, *P. ovale*, *P. vivax*, *P. falciparum* and *P. malariae*. Four µL of nested 1 PCR product was used as the template for each of nested 2 PCR assays. The conditions and concentrations used in the nested 2 PCR amplifications were similar to those used in nested 1 PCR, except for the annealing temperature (58°C) for species-specific primers (PkF1140/PkR1550, rOVA1/rOVA2, rVIV1/rVIV2, rFAL1/rFAL2 and rMAL1/rMAL2). The amplified products were sent to MyTACG Bioscience Enterprise for sequencing to validate the identities of the species. On top of that, Loop-Mediated Isothermal Amplification Assay (LAMP) was performed to confirm doubtful or inconclusive results produced by

Table 1. Sequences of primers used in this study

Species	Name of primer	Sequences (5'-3')	Annealing temperature (°C)	Product size (bp)	References
<i>Plasmodium</i> spp.	rPLU1	TCAAAGATTAAGCCATGCAAGTGA	55	1200	Snounou <i>et al.</i> , 1993
	rPLU5	CCTGTTGTTGCCCTAAACTCC			
<i>P. knowlesi</i>	PkF1140	GATTCATCTATTAATAAATTGCTTC	58	410	Imwong <i>et al.</i> , 2009
	PkR1550	GAGTTCTAATCTCCGGAGAGAAAAGA			
<i>P. ovale</i>	rOVAL1	ATCTCTTTTGCTATCTTTTTTAGTATTGGAGA	58	659-662	Snounou <i>et al.</i> , 1993
	rOVAL2	GGAAAAGGACACATTAATTGTATCCTAGTG			
<i>P. vivax</i>	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	68	121	Snounou <i>et al.</i> , 1993
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA			
<i>P. falciparum</i>	rFAL1	TTAAACTGGTTTGGGAAAACCAATATATT	58	206	Snounou <i>et al.</i> , 1993
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC			
<i>P. malariae</i>	rMAL1	ATAACATAGTTGTACGTAAAGAATAACCGC	58	145	Snounou <i>et al.</i> , 1993
	rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA			

microscopy and nested PCR. LAMP was carried out according to the previous publication (Lau et al., 2016). The amplification was carried out in a Loopamp real-time turbidimeter (LA-320; Teramecs, Co., Ltd., Tochigi, Japan).

RESULTS

From the initial microscopy examination, 9, 60, 25, 6 and 11 samples were diagnosed as *P. falciparum*, *P. knowlesi*, *P. vivax*, *P. malariae* and mixed infections (*P. falciparum*/*P. vivax*, *P. knowlesi*/*P. falciparum* and *P. knowlesi*/*P. malariae*), respectively (Table 2). Out of the 11 mixed infections, 1 sample was negative for malaria infection, 2 samples were screened as single infection for *P. falciparum* and 8 samples were positive for *P. knowlesi* using nested PCR. Nested PCR detected 15 additional *P. knowlesi* on top of the 39 detected by microscopy, apart from the 8 *P. knowlesi*/*P. malariae* mixed infections, 1 was initially diagnosed as *P. falciparum*, and 3 as *P. vivax* and *P. malariae*, respectively.

The samples were collected from district or states hospitals or clinics from Kuala Lumpur, Pahang, Perak, Pulau Pinang, Terengganu, Sabah and Sarawak throughout the study (Figure 1). By using the previously published primer sequences, the size of the amplicons generated by *P. knowlesi*, *P. ovale*, *P. vivax*, *P. falciparum* and *P. malariae* were 410 bp, 659-662 bp, 121 bp, 206 bp and 145 bp, respectively (Figure not shown). Out of 112 samples, only 80 cases were positive for malaria infections following the nested PCR assays screening (Table 3). In Pulau Pinang, *P. knowlesi*, *P. vivax* and *P. falciparum* were responsible for 4.4% (n=5), 7% (n=8) and 3.5% (n=4), respectively. Meanwhile, in Perak, 1 case of *P. malariae* was recorded and it was the only one incidence (0.9%) was recorded during the sample collection period. In Pahang, total of 6 cases (5.3%) were found and all samples were positive for *P. knowlesi*. Meanwhile, out of 47 cases in Sarawak, 30 samples (26.5%) were positive for *P. knowlesi* and the rest of the samples were negative for malaria. In Kuala Lumpur, *P. vivax* and *P. falciparum* responsible for 2.7% (n=3) and 1.8%

Table 2. Detection of *Plasmodium* spp. by microscopy and PCR methods in malaria patients samples

	Result of microscopy examination									No. cases by PCR
	<i>Pf</i>	<i>Pk</i>	<i>Pv</i>	<i>Pm</i>	<i>Po</i>	<i>Pk/Pm</i>	<i>Pf/Pv</i>	<i>Pk/Pf</i>	Negative	
<i>Pf</i>	4	0	1	0	0	1	1	0	0	7
<i>Pk</i>	1	39	3	3	0	8	0	0	0	54
<i>Pv</i>	1	1	9	0	0	0	0	0	1	12
<i>Pm</i>	0	1	0	3	0	0	0	0	0	4
<i>Po</i>	0	0	0	0	0	0	0	0	0	0
<i>Pk/Pv</i>	0	0	3	0	0	0	0	0	0	3
Negative	3	19	9	0	0	0	0	1	0	32
Total	9	60	25	6	0	9	1	1	1	112

Pf: *P. falciparum*; *Pk*: *P. knowlesi*; *Pv*: *P. vivax*; *Pm*: *P. malariae*; *Po*: *P. ovale*.

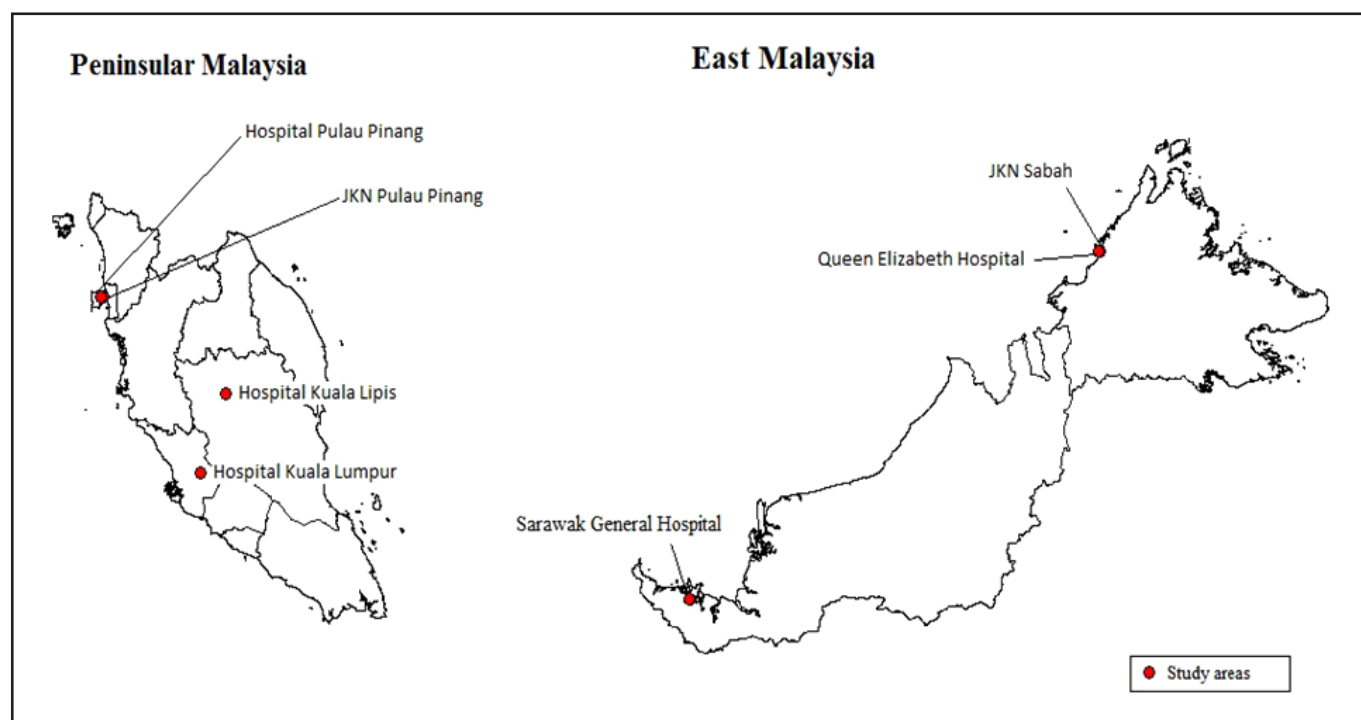
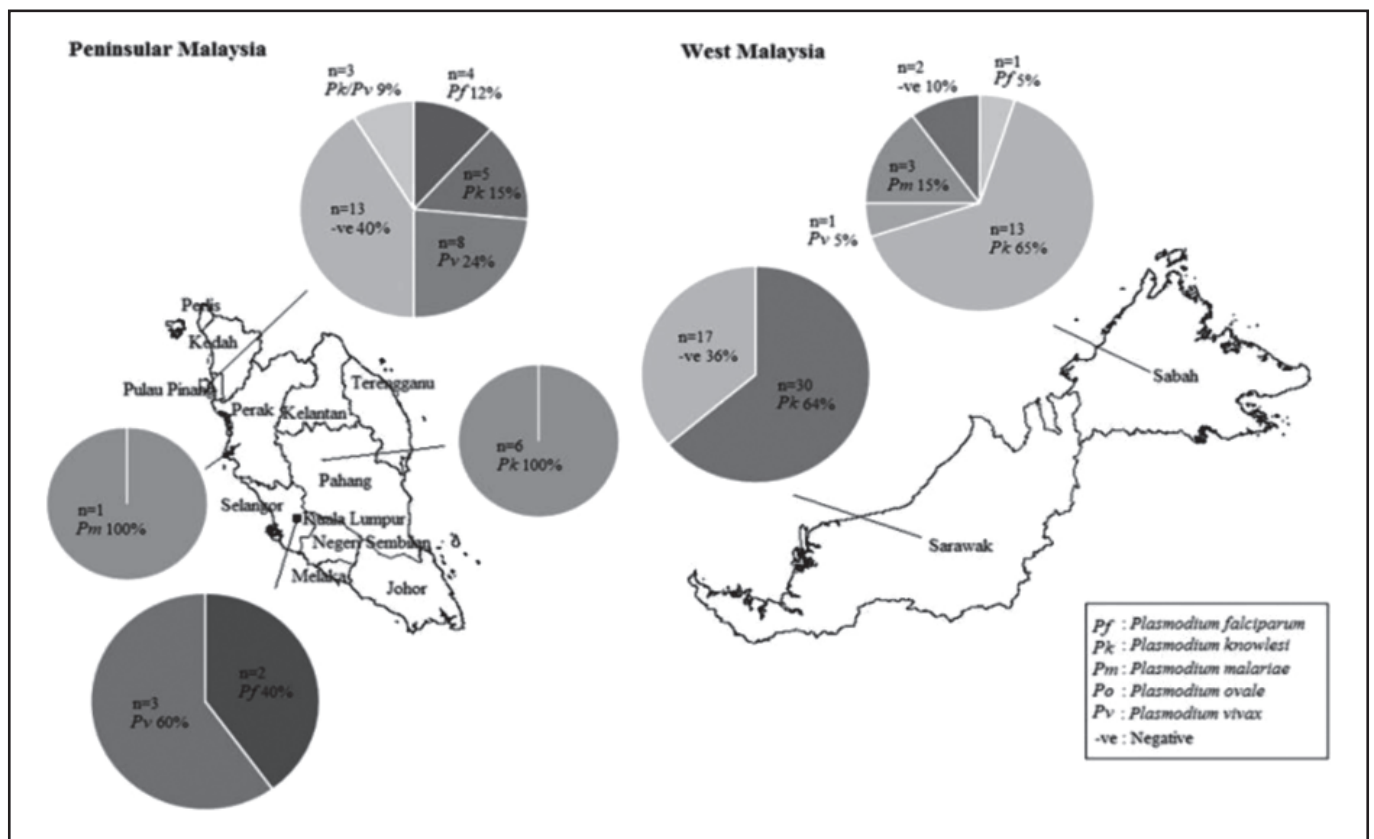


Figure 1. Map of Malaysia showing the locations of study.

Table 3. PCR-based species profile of malaria cases collected from areas in the present study

State	<i>P. falciparum</i>	<i>P. knowlesi</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>	<i>P. knowlesi/ P. vivax</i>	Negative	Total
Kuala Lumpur	2	0	3	0	0	0	0	5
Pahang	0	6	0	0	0	0	0	6
Perak	0	0	0	1	0	0	0	1
Pulau Pinang	4	5	8	0	0	3	13	33
Terengganu	0	0	0	0	0	0	0	0
Sabah	1	13	1	3	0	0	2	20
Sarawak	0	30	0	0	0	0	17	47
Total	7	54	12	4	0	3	32	112

**Figure 2.** Map of Malaysia showing *Plasmodium* spp. areas. Pie chart represents the number of cases and percentages of each *Plasmodium* spp. and negative malaria infections determined by species specific nested PCR.

(n=2), respectively. In Sabah, *P. knowlesi*, *P. vivax*, *P. falciparum* and *P. malariae* were responsible for 11.5% (n=13), 0.9% (n=1), 0.9% (n=1) and 2.7% (n=3), respectively. However, no cases were positive for any of the *Plasmodium* spp. nested PCR assays in Terengganu (Figure 2). Three mixed infections of *P. knowlesi/ P. vivax* were also detected in Pulau Pinang. Among the 112 samples, no samples were positive for *P. ovale* nested PCR assays. A total of 32 microscopy positive samples were not detected using nested PCR. Some of the false positive and false negative results were double confirmed with LAMP.

DISCUSSION

This study highlights the incidence of malaria infections from Kuala Lumpur, Pahang, Perak, Pulau Pinang, Terengganu, Sabah and Sarawak with exclusively attention in *P. knowlesi*

malaria. The collected data shows that *P. knowlesi* achieved the highest prevalence among other human malaria *Plasmodium* spp. with 54 cases (48.2%) between year 2015 and 2016. In comparison to *P. knowlesi* infection, a small number of *P. falciparum* (7 cases or 6.3%) and *P. vivax* (12 cases or 10.7%) malaria were obtained from the data collection areas. In 2014, the previous study showed that *P. falciparum* and *P. vivax* malaria in Malaysia were 11% and 29%, respectively (Yusof et al., 2014). This has coincided with a decline of both cases in Malaysia. Moreover, this trend proved the successful of the malaria eradication program.

From the collected data, 32 cases were not detected by PCR. The patients presented quotidian pattern of febrile illness such as daily fever and chills during the admission to the hospitals. These patients were enrolled in our study as they were microscopy positive for malaria infection.

Nonetheless, the PCR results screening shown negative for *Plasmodium* spp. These samples were stored a few months before shipping to the laboratory for further PCR diagnosis. Prolonged storage of the samples while waiting to be sent also contribute to the possibilities of negative PCR results. On top of that, the DNA may be degraded while shipping at room temperature to the laboratory. Shipping over long distance may cause the loss of the DNA quality. Therefore, efficient and reliable transport is crucial to avoid loss in DNA quality of the samples (Straube & Juen, 2013).

From Table 3, three cases of *P. knowlesi* infection were misdiagnosed as *P. malariae* and 1 as *P. falciparum* by microscopic examination. *P. knowlesi* was misdiagnosed as *P. malariae* due to their similarities of morphology. Early trophozoites of *P. knowlesi* were similar to *P. falciparum* while other stages were resemble to *P. malariae* (Sulistyarningsih et al., 2010). In fact, macaques are a natural reservoir for *P. knowlesi* and other simian *Plasmodium* spp.. In Malaysia, researchers begin focus on the investigation of this parasite when human infections on *P. knowlesi* was largely reported from Kapit, Sarawak in 2004 (Singh et al., 2004). Comprehensively, researchers should pay extensive attention on any potential incidence presenting 'P. falciparum-like parasites' and 'P. malariae-like' parasites. Misdiagnosed *P. knowlesi* as *P. falciparum* may delay the treatment and potentially caused fatal to the patients due to the rapid replication cycle of *P. knowlesi*.

The present study indicates that *P. knowlesi* was the highest prevalence (55.6%) in Sarawak. This has been coincided with a previously published report that indicated the highest incidence of *P. knowlesi* malaria from Sarawak as well (Yusof et al., 2014). The finding revealed that *P. knowlesi* has a higher proportion to cause significant public health problems among the populations in Sarawak. The highest incidence of the zoonosis *P. knowlesi* in Sarawak may due to the deforestation and logging activities in certain interior regions. As forest was the natural habitat for macaques, deforestation activities would lead their movement to the areas near housing environment. Thus, the chances of parasites transmissible from macaques to humans via mosquito bites were increased. The *P. knowlesi* which was initially with macaques as the reservoir host may switch to humans as the preferred hosts. Therefore, effective control, prevention and management system is necessary in order to reduce the morbidity and mortality rates among the populations in Sarawak.

Though microscopy is a gold standard for the detection of malaria, our findings suggest that nested PCR is more specific in detection of *P. knowlesi*. Thus, nested PCR was a highly recommended method in diagnosis especially in the detection of samples with mixed infections and low parasitemia as microscopic examination may cause the likelihood of false negative results. Therefore, microscopic alone was not reliably to distinguish *P. knowlesi* from *P. malariae*, *P. falciparum* and *P. vivax*.

In this study, PKF1140 and PKR1550 primer pairs (~450 bp) was used for *P. knowlesi* species-specific diagnosis in nested 2 PCR while the commonly used pmk8 and pmkr9 primer pairs were not used. Imwong et al. (2009) has proved that non-specific binding was found between *P. knowlesi* and *P. vivax*. Although the forward sequence, pmk8 was highly specific to *P. knowlesi*, the reverse sequence, pmkr9 was found in other *Plasmodium* spp. especially *P. vivax* S-type strains.

Malaysia has declared its aim to eradicate malaria infection by year 2020. To achieve this compliment effectively, there is necessary to investigate the route of the true

transmission of malaria infections. It has been proposed that there were three possible ways of transmission; macaques-human, human-human or human-macaques. Interruption of the transmission pathway is the prerequisite in successfully elimination of malaria. Understanding the risk factors of malaria infections and mosquito vectors may assist in control and elimination of human malaria.

In conclusion, higher levels of *P. knowlesi* infection has challenged the malaria eradication efforts in Malaysia. Among the *Plasmodium* spp., *P. knowlesi* caused the highest prevalence of infections, specifically in Sabah and Sarawak. Meanwhile, the highest prevalence of *P. knowlesi* in Peninsular Malaysia was noticeable in Pulau Pinang. To limit the further increase of the *P. knowlesi* infections in Malaysia, it is crucial to implement an effective control and management strategy as well as understanding the malaria vector (*Anopheles* spp.) behaviour around the malaria endemic areas.

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Conflict of interest

The author declares that they have no conflict of interests.

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